EPIDEMIOLOGICAL AND IMMUNOLOGICAL INVESTIGATIONS IN STREPTOCOCCAL DISEASES

by

Tats Yamamoto

PLATE I

Guinea-pig eye lesion showing the
sero fibrinous film covering the conjunctiva

ABSTRACT

Section I

A study is made of an epizootic lymphadenitis in guinea-pigs which occurred at the Provincial Laboratory of Public Health, Edmonton, Alberta. The investigation included the study of various control methods, bacteriology of the infecting organism, mode of transmission, and variations in the virulence of the disease.

The disease was characterized by an explosive outbreak of an acute, rapidly spreading infection affecting a large proportion of animals and decimating whole herds. The first sign of infection in the animals was an inflammation of the conjunctiva followed by cervical adenitis exhibiting large pus-filled nodes. Control of the disease was successfully effected by removal of all animals, disinfection of equipment and rooms, and use of a skin hypersensitivity test on fresh stocks of animals. The propagation of an experimental herd of naturally infected animals produced only the chronic phase of the disease, characterized by enlarged lymph glands and a low mortality.

Production of the disease by artificial means was studied. Intraperitoneal inoculation produced an acute disease resembling that found in the natural epizootic. Conjunctival inoculation induced the development of chronic cervical adenitis. The experiment on aerial transmission showed that the disease was not spread by air in the chronic phase.

The infecting organism was Streptococcus pyogenes epizooticus which was found to belong to Lancefield's Group C when precipitin tests were carried out on organisms lyzed by extracts of Streptomyces albus.

The lack of streptolysin O production by this streptococcus prevented the use of the antistreptolysin test as a disease control measure. The investigation of this test, however, led to the study of streptolysin O and the subsequent study of human antistreptolysin O levels.

Section II

The desirability of the application of the antistreptolysin O test as a routine diagnostic test in certain human diseases necessitated the preparation and maintenance of antigenically active streptolysin O. Antigenically active streptolysin O maintained in the refrigerator in the reduced state was found to retain its potency.

This investigation was followed by a study of the antistreptolysin O response in various streptococcal diseases occurring in the Edmonton region. Elevated antistreptolysin O titres were found in nearly all cases of rheumatic fever and glomerulonephritis, while low titres were generally found in the non-streptococcal disease group. Antistreptolysin O titres of newborn infants were in most cases found to be higher than their mothers' titres.

THE UNIVERSITY OF ALBERTA

EPIDEMIOLOGICAL AND IMMUNOLOGICAL INVESTIGATIONS IN STREPTOCOCCAL DISEASES

I.

A STUDY OF A STREPTOCOCCAL EPIZOOTIC IN GUINEA-PIGS

II.

THE PREPARATION OF A STABLE ANTIGEN FOR THE ANTISTREPTOLYSIN O TEST AND A STUDY OF LOCAL ANTISTREPTOLYSIN O LEVELS

A DISSERTATION

SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES

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SECTION I

A STUDY OF A STREPTOCOCCAL EPIZOOTIC IN GUINEA-PIGS

INTRODUCTION

Although the study of bacterial diseases of humans is often thought more important than any study of bacterial diseases of animals, a knowledge of the mechanism and treatment of the latter is often of fundamental importance. Besides the fact that we depend upon various domestic animals as a food source and therefore are interested in their well being, many human diseases have their analogues in animal diseases and these offer an approach to the experimental study of these disease processes.

Among such diseases common to humans and animals infections with Salmonellae are probably more widespread than infections with any other group of bacteria. They are known to infect most species of wild and domestic animals and birds. Among the rodents Salmonella typhi-murium and S. enteritidis are often the infecting agents. Horses are often infected with S. abortus equi causing infectious abortion of mares. Domestic fowls are commonly infected with members of the Salmonella group causing large economic losses.

Other bacterial diseases are also prevalent among animals. Staphylococcus mastitis in cattle is not uncommon and is spread by the milker from animal to animal as well as to humans. Brucellosis among

goats, cattle and swine is an important disease from the public health point of view because of the ease of transmissibility of the disease from animals to man. Plague is primarily a disease of rats and other rodents and is spread from rat to rat and from rat to man through the bite of infected fleas. Among the genus Corynebacterium are a number of species known to affect animals. Notable among these is C. ovis or C. pseudo-tuberculosis, which produces a caseous lymphadenitis and ulcerative lymphangitis in sheep and horses referred to as pseudotuberculosis. True tuberculosis of lower animals living under natural conditions is very rare but is not uncommon in domestic animals. Among the streptococci are a number of strains responsible for specific diseases of domestic animals. Streptococcus equi is the cause of strangles in horses. A common cause of mastitis in cattle is S. agalactiae.

Many of these organisms are not transmissable to man though they belong to species causing human disease, but a study of such infections can still yield valuable lessons to the experimental pathologist or epidemiologist. The present study of an explosive epizootic, a strepto-coccal lymphadenitis, among laboratory guinea-pigs was contracted with the primary purpose of dealing with a current emergency but also with the hope of obtaining basic epidemiological information. Although this hope has not been fully realized the investigation has determined the nature of the infecting organism and the method of transmission and gives an account of successful immediate and long-term control measures.

The study of streptococcal lymphadenitis among guinea-pigs was initiated as a result of an explosive epizootic infecting a large portion of the guinea-pig population at the Provincial Laboratory of Public Health, Edmonton, Alberta. As new lots of guinea-pigs were received, the

disease was transmitted to them in a few weeks' time, decimating the number of healthy guinea-pigs. The eradication of the streptococci became a major problem due to the high morbidity of the infecting strain. The purpose of this investigation was to attempt to determine the nature of the infecting organism, the method of transmission of the organism and control measures to prevent the spread of the organism.

LITERATURE REVIEW

STREPTOCOCCAL INFECTION OF GUINEA-PIGS

The first account of spontaneous streptococcal infection in guinea-pigs was by Eberth in 1885 who described a chronic streptococcal infection invading the liver, spleen and lungs. Teacher and Burton (1914) described an epizootic caused by streptococci which produced infectious abortion. Holman (1916) stated that the principal disease caused by streptococci was pneumonia, but he also found that uterine infections were common. Boxmeyer (1907) was the first to describe a large epizootic characterized by cervical lymphadenitis. His excellent description could be applied to the reports of most subsequent workers except for minor variations characteristic of each particular epizootic. The streptococcal disease studied in this thesis is similar in nature to that of Boxmeyer.

Epizootic Lymphadenitis in Guinea-pigs

The disease is characterized by great enlargement of the cervical lymph nodes which contain large quantities of pale-yellow, granular pus. The pus is said to have the consistency of "schmierkase." Microscopically the pus is found to contain large numbers of polymorphonuclears in various stages of disintegration. Gram stained smears show many Gram-positive cocci as well as many degenerate forms of cocci. These abscesses according to Boxmeyer (1907) vary from 1 to 12 in number and are 15 to 50 millimeters in diameter. The abscesses are loosely attached to the adjacent structure and have thick capsular walls over which run dilated blood vessels.

Other organs are often affected. Lesions can be found in the heart, liver and spleen. In the epizootic described by Rae (1936) the most constant feature of infection was an enlargement of the myocardium and small elevated, yellowish areas on the pericardial surface and ventricle.

In its most chronic form the disease can persist in a herd for years with only an occasional guinea-pig showing signs of "lumps." In its most acute form the disease produces a rapidly fatal septicemia and spreads through a herd very rapidly. Seastone (1939) reports a spontaneous variation from the chronic to the acute phase in an infected herd.

BACTERIOLOGY OF EPIZOOTIC LYMPHADENITIS

In every epizootic reported the incriminated organism is a hemolytic streptococcus. These streptococci can be isolated from the swollen lymph nodes, pericardial and myocardial lesions, liver and kidney. In animals dying of septicemia the organism can be recovered from the blood. The organism tends to be capsulated in young cultures and grows as a somewhat viscid colony, rather larger than the usual Group A streptococcus colony.

Although hemolytic streptococci are constantly found associated with the production of epizootic lymphadenitis they may be accompanied by other organisms. Megrail and Hoyt (1929) stressed the frequency of occurrence of Staphylococcus albus as a secondary invader in the lymph glands, an observation which has been supported by other workers. All experimental work, however, has confirmed the essential importance of the hemolytic streptococcus in the production of the characteristic

disease.

The Identity of the Infecting Streptococcus

Ever since Rosenbach in 1884 named the coccoid micro-organism of human infectious disease Streptococcus pyogenes, the nomenclature of the various species within the genus has been varied and confusing. According to Cunningham (1929) the streptococci associated with epizootic lymphadenitis fell into Holman's Streptococcus anginosus group. Later it was referred to as "animal" pyogenes without any formal name. According to Lancefield (1928) the organism belongs to Group C. The immunological classification which she used is most useful from the standpoint of disease inducing capacities and animal species affected. This classification is dependent upon the presence of a group specific carbohydrate C as shown by Lancefield (1928, 1933, 1941).

The various strains in Group C have been difficult to classify formally. The organisms falling in "animal" Group C and found in animals are designated as "animal" Streptococcus pyogenes, Streptococcus pyogenes animalis and Streptococcus zooepidemicus. Swift, in the textbook "Bacterial and Mycotic Infections of Man, page 289 (1952) suggests that the term Streptococcus pyogenes epizooticus most accurately describes the "animal" Group C strain. This name is used in this thesis for the organism causing lymphadenitis in guinea-pigs.

In the classification of the various strains within Group C both the serological grouping technique and the physiologic tests are important. The hemolytic strains of Group C can be divided into three distinct varieties based on their action on trehalose, sorbitol and glycerol. Streptococcus equi and "animal" pyogenes are separated from

other hemolytic streptococci by their failure to ferment trehalose and glycerol and are separated from one another because Streptococcus equi does not ferment sorbitol while the "animal" pyogenes does ferment sorbitol.

Certain strains of Group C are easily confused with Group A and large colony Group G, their definite identification rests upon the serologic method.

Serological Typing of Group C Hemolytic Streptococci

The Group C hemolytic streptococci can be divided into types. The type specific antigen appears to be of protein nature and there are 5 main types. On the basis of fermentation of trehalose and sorbitol this group is differentiated into three subgroups by Edwards (1934). According to Bazeley and Battle (1940) all strains of the first subgroup fall into one serological type. The second subgroup does not ferment trehalose but does ferment sorbitol. In this subgroup there are two serological types, distinguished biochemically because one type ferments lactose and the other does not. The third subgroup ferments trehalose but not sorbitol and also has two serological types, type 4 and type 5. Type 5 is found in humans and it is distinguished from type 4 by its failure to ferment lactose. A summarized table (Table No. I) is shown with the relationship of subgroup, type and fermentation reactions. According to this table Streptococcus pyogenes epizooticus as the causative organism of epizootic lymphadenitis of guinea-pigs is likely to belong to Type 2.

TABLE I

Fermentation and Type of Group C Hemolytic Streptococci

Subgroup	Type	Habitat	Trehalose	Sorbitol	Lactose	Salicin
1	1	horses (strangles)	-	-	-	+
2	2	horses, guinea-pigs, cattle, rabbits	-	+	+	
	3	horses (equine respiratory catarrh)	-	+	-	
3	4	horses	+	-	-	+
	5	humans (human "C")	+	-	+	+

EPIDEMIOLOGY

Mode of Transmission

There is no general agreement by the various authors as to the mode of spread of the streptococci from one animal to another. Following is a list of methods by which the organisms were thought to spread: -

1. Upper digestive tract through abrasions in the mouth.
   Boxmeyer, (1907)
2. Upper respiratory tract, middle ear, and mouth.
   Branch (1927).
3. Skin scratching and biting of ears.
   Megrail and Hoyt (1929).
4. Genitalia.
   Hardenbergh (1926), Parsons and Hyde (1928).
5. Nasopharyngeal secretions.
   Rae (1936).

6. Ingestion of infective agent.

Seastone (1939).

In most cases it was agreed that spread by contact was more important than spread by air droplets.

Pathogenicity

In its most chronic form the disease can persist in a herd for years with the occasional guinea-pig showing signs of "lumps." The number of guinea-pigs affected is difficult to assess because the symptoms are slight. Mortality appears to be low or non-existent in this chronic form with the infected animals recovering completely.

In its most acute form the disease produces a rapidly fatal septicemia which spreads through a herd very rapidly. Variation in virulence was found by Seastone (1939) in the strains he isolated from guinea-pigs. He reports a chronic form of the disease in a herd which lasted for a period of 7 years when suddenly about half the animals died in a month's time. During this period he was able to isolate both strains, the chronic and virulent forms of the streptococcus, from the heart blood. The virulent strain was associated with larger capsules and longer chains than the chronic strain. After the acute epizootic had abated the chronic disease still remained and only the "chronic" strain could be isolated from the infected animals. Seastone ascribed the appearance of the virulent strain in the herd to a spontaneous variation of the chronic strain. He was able to differentiate 8 different strains of the streptococcus according to colonial morphology, virulence, capsule production and growth characteristics.

Rae (1936) described an outbreak among stock guinea-pigs which assumed epizootic proportions soon after its appearance but which gradually subsided to the occurrence of a few cases every month for over a period of one year.

Experimental production of disease has been attempted by several workers. They reported the production of disease processes similar to those found in naturally infected guinea-pigs. Cunningham (1929) found that rabbits died in a few days following intravenous injection with broth cultures. Mice injected intraperitoneally with small doses of the organism were found to develop a fatal mucilaginous, fibrinous, adhesive peritonitis.

Development of Hypersensitivity

The development of cutaneous hypersensitivity by infected animals was studied by Moen (1936). He followed a technique similar to the tuberculin test, using extracts from Group C hemolytic streptococci. The following reactions occurred: -

1. All animals infected with Group C hemolytic streptococci gave a positive reaction to the bacterial extract.

2. Animals recently recovered from the streptococcal infections gave positive reactions of diminished intensity.

3. Animals not reacting to the extract were free of Group C hemolytic streptococcus infection.

He suggested that skin testing with a bacterial extract prepared from a Group C streptococcus offered a method of detecting guinea-pig streptococcal carriers even where they showed no obvious external evidence of the infection. He also suggested that the skin test could be used as a means of obtaining a guinea-pig stock free of hemolytic streptococcus causing lymphadenitis.

RESULTS

ACUTE NATURAL EPIZOOTIC

Occurrence

The disease was introduced into the Provincial Laboratory on August 7, 1951 when 68 guinea-pigs were bought from a local dealer. It is not known what proportion of animals was infected on arrival at the Laboratory nor were there records kept of the number of animals that subsequently succumbed to the disease. This acute disease spread very rapidly to all other guinea-pigs brought in to the Laboratory, decimating the numbers to such a point that the availability of guinea-pigs for diagnostic tests was seriously reduced. Also, even when presumably healthy guinea-pigs were used for laboratory tests many of them succumbed to the streptococcal disease before the completion of the tests. The epizootic lasted for approximately 10 months and at the end of that time the disease was successfully eliminated by drastic control measures.

Clinical Features

The acute disease among the guinea-pigs began with an explosive outbreak and rapidly assumed epizootic proportions. The disease appeared to spread by contact through both the old and new stock with fatal results to a large portion of animals. Age or sex of the animals did not appear to influence their susceptibility to the disease.

The first sign of infection of the guinea-pig was inflammation of the conjunctiva. This inflammation would persist for 3-4 days and was followed by a gradual development of a thin serofibrinous film over the eye and infection of the conjunctival and scleral vessels. This was followed in about another 3 days by edema and protrusion of the eye.

Several weeks after the appearance of the eye lesion the disease was seen to take different courses in the animals. In some animals there was complete resolution with no complications. Other guinea-pigs went blind and eventually died. Some animals developed pneumonia, while others developed septicemia. These also died in a very short period. Many of the animals developed lymphadenitis following the eye lesion. This was one of the more characteristic features of the disease. This phase of the disease was of a chronic nature, the abscesses which formed often persisting for weeks and months without rupture.

Description of the Infected Lymph Glands

The proportion of animals with abscessed neck glands in the infected herd varied anywhere from 30% - 70%. The nodes varied in size from 10 mm. to about 50 mm. in diameter and as many as 4 or 5 of them might be found on the neck of a single animal. The nodes were loosely attached to the body but often firmly fixed to the adjacent skin. Examination of an infected lymph node usually revealed a well demarcated abscess surrounded by a stout fibrous capsule quite difficult to cut through. Histological preparations showed tissue changes of chronic inflammatory type. The abscess cavity was filled with thick, granular, pale-yellow pus.

In the adult guinea-pig the abscess generally enlarged to about 25 mm. in diameter and then ruptured discharging the pus throughout the cage in which the animal was housed. The guinea-pig did not seem to be particularly distressed, and after the discharge of pus the lesion often healed completely leaving a small scar at the point of rupture.

Bacteriology

Cultures made from numerous guinea-pig autopsies showed hemolytic streptococci to be present in the blood and internal organs of the animals. Repeated culture of pus taken from the cervical glands and of swabs from the conjunctival lesions produced similar streptococci.

These streptococci were hemolytic on blood agar, producing large, clear zones of hemolysis surrounding the fine, clear colonies. Precipitin tests on extracts of the streptococci showed that the organism belonged to Lancefield's Group C. This classification was first established by the Laboratory of Hygiene, Ottawa, but subsequent tests were done at this Laboratory with antisera received from Ottawa.

Fermentation tests showed that the organism fermented sorbitol but not trehalose, thus indicating that it belonged to "animal" Group C.

Immediate Control

When the disease was first detected measures were instituted which on previous occasions had been sufficient to eliminate infections of this nature. Stock animals were inspected at frequent intervals and all animals with enlarged cervical nodes were eliminated. Later, because of increased familiarity with the clinical features of this epizootic, animals showing evidence of conjunctivitis or even of trivial ill health were ruthlessly sacrificed. Cages, racks and all utensils which had come in contact with infected animals were carefully disinfected and various schemes of isolation or segregation of all animals which had been in contact in any way with the infection were put into operation. Fresh healthy stocks were introduced into separate, especially disinfected rooms and an attempt was made to keep all operations of cleaning, feeding and attendance

on these entirely separate from similar operations on the other animals which had been exposed. All these measures met with some success, the infection would abate for a few weeks and then a new case would appear in healthy stocks or in "clean" rooms. No matter how promptly this case was eliminated successive cases arose and rapid deterioration of the epidemiological situation occurred. Cycles of epizootic prevalence and apparent control ensued and it soon became apparent that none of the above measures was going to be successful. More drastic steps had to be taken.

The final control operations were simple. All stock guinea-pigs were destroyed with the exception of a few removed to the Department of Bacteriology to establish an experimental herd. All inoculated animals were removed to a basement room of the Provincial Laboratory where they were looked after by attendants entirely separate from those working in the usual animal quarters. The regular animal rooms were then emptied of everything movable and scrubbed out with Zycol* on three separate occasions once a week. Metal cages and racks were dismantled and boiled. Wooden stock cages were repeatedly scrubbed with Zycol. Culture plates were exposed in all areas to confirm the absence of the epidemic strain. When these procedures had been completed satisfactorily, fresh stock was brought in. Each batch of new guinea-pigs was maintained in isolation under observation until its freedom from infection could be established and until by inference the breeder "source" could be considered "clean." Skin tests with streptococcal antigen were performed on many of these animals. Details of these tests will be given later. Inoculated animals from the original stock were maintained in their basement quarters under complete isolation until they died from intercurrent streptococcal infection or were killed off on completion of the diagnostic tests.

* Zycol is a saponified creosol supplied by Alberta National Drug Co.

By these drastic control measures the disease was successfully eliminated from the Provincial Laboratory. No sign of a streptococcal disease of a similar nature has been observed there for a period of 3 years since the introduction of control measures.

STUDY OF EXPERIMENTAL HERD

Introduction

Further study on the nature of the disease and the infecting organism was attempted by the propagation of an experimental naturally infected herd of animals. When the infected animals were being destroyed at the Provincial Laboratory approximately 15 animals were brought to the Department of Bacteriology where a herd of naturally infected animals was kept from May 1952 to October 1954.

Maintenance

The herd usually numbered between 6 and 15 animals and was maintained by the addition of normal guinea-pigs from time to time. By this method the disease was found to propagate naturally and inoculation of the animals was not necessary.

PLATE II

Pyogenic lesion in spleen of guinea-pig

from which hemolytic streptococci were obtained

PLATE III

Guinea-pig heart showing streptococcal lesion

situated in the ventricle

PLATE IV

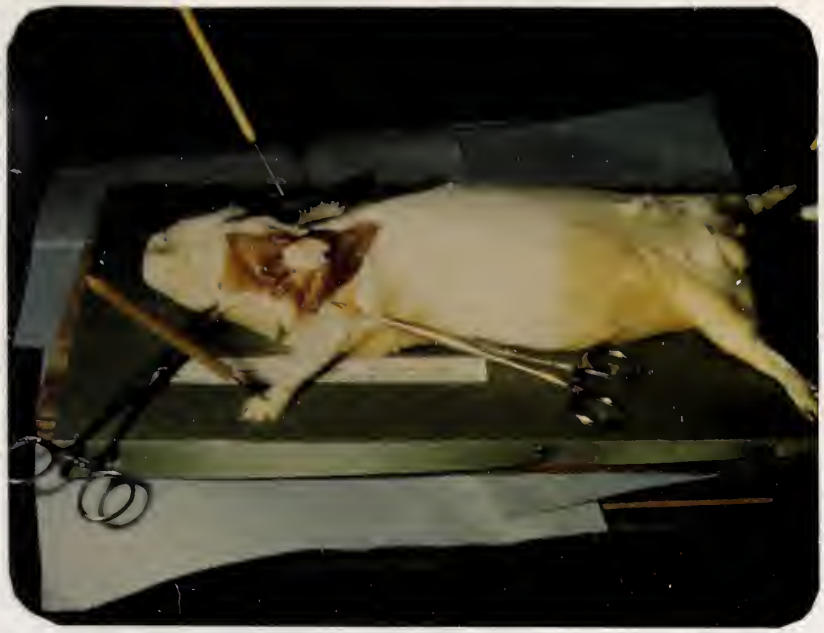
Guinea-pig with large lymph gland.

The lower node has ruptured and the pus has drained away.

PLATE V

Guinea-pig lymph node with adjacent skin cut away.

PLATE VI



The abscess cut open to reveal the cheesy pus inside

PLATE VII

Guinea-pig with adjacent skin removed from 4 large abscesses.

One abscess has been punctured.

Clinical Features

By the time the experimental herd was set up a change in the nature of the disease appeared to have taken place. The chronic lymphadenitis form was common and acute episodes were rare.

The disease in this herd was characterized by low mortality and comparatively slow disease processes as compared to that found in the original epizootic. The only sign of infection was the formation of "lumps" in the cervical region. Many animals carried these swellings for many weeks before they ruptured. After rupture, the lesions healed leaving only small scars. The presence of 1 - 4 large lymph node swellings did not appear to affect the animals in any way. When the "lumps" disappeared, no further signs of infection were observable. The appearance of the lymph glands in this group of animals was similar in all respects to those found in the natural epizootic.

The acute form of the disease as found in the natural epizootic was totally absent. Even the conjunctival lesions which were characteristic of the epizootic were not observed. Although it was hoped that the acute phase of the disease would reappear naturally due to a bacterial variation of the infecting strain, there was no change in the chronic nature of the disease in the experimental herd.

Bacteriology

A number of guinea-pigs in the experimental herd were examined for the presence of pathogenic bacteria. A total of 18 autopsies were performed on guinea-pigs from this herd. Four animals with chronic lymphadenitis were killed for examination, while 14 animals died during the duration of the experiment.

From the 4 animals killed, only hemolytic Group C streptococci were isolated. These organisms were found only in nodes. No other organisms were isolated from any tissue or organ.

From the 14 animals that died, other organisms besides streptococci were isolated in nearly every instance. The bacteriological findings are shown in Table II. A paracolon bacillus was found in 3 animals but no hemolytic streptococci were found in two of them. The two organisms most consistently found together were the hemolytic streptococci and Staphylococcus albus. These organisms were isolated from 7 animals.

Although the hemolytic streptococcus was present in nearly all the animals examined, it is possible that a few animals succumbed to the combined action of several species of bacteria.

It seems likely that even the most chronic form of streptococcal lymphadenitis can decrease general resistance and thus favour invasion with other bacteria.

The streptococci isolated from these guinea-pigs appeared to be quite similar in nature to those found in the original epizootic. The morphological and cultural characteristics of all these streptococci will be discussed more fully later on.

TABLE II

Bacteriological Findings

on Autopsies of Guinea-pigs from the Naturally Infected Herd

No.	Result	Bacteriological Finding
1	Killed	Hemolytic streptococci from lymph node only
2	Died	Hemolytic streptococci from lungs; hemolytic staphylococci from lesion on back
3	"	Hemolytic streptococci in blood and lymph gland
4	"	*Streptococcus viridans* and *Staphylococcus albus* in blood and lymph gland
5	"	*Streptococcus viridans* and *Staphylococcus albus* in blood and lymph gland
6	"	*Streptococcus viridans* and *Staphylococcus albus* in blood and lymph gland
7	"	Paracolon in blood
8	"	Hemolytic streptococci in lymph node; mixed flora in lungs
9	"	Hemolytic streptococci in lymph node and lungs; paracolon in lungs
10	"	Paracolon in blood
11	"	Hemolytic streptococci in lymph node
12	Killed	Hemolytic streptococci in lymph node
13	"	Hemolytic streptococci in lymph node
14	"	Hemolytic streptococci in lymph node
15	Died	Hemolytic streptococci and *Staphylococcus albus* in blood
16	"	Hemolytic streptococci and *Staphylococcus albus* in blood
17	"	Hemolytic streptococci and *Staphylococcus albus* in lymph node
18	"	Hemolytic streptococci and *Staphylococcus albus* in blood

ARTIFICIAL PRODUCTION OF DISEASE

Intraperitoneal Inoculation

Several workers have shown that various phases of the disease can be reproduced by inoculating animals subcutaneously, intraperitoneally, or intravenously. Rae (1936) reported the enlargement of lymph nodes within 4 days after the above inoculations and the production of an acute disease with death within 12 days. The most consistent findings were those in the heart. In nearly every animal she found numerous soft yellowish lesions in the myocardium similar to the lesions in the spontaneously infected animals which she investigated.

In this investigation experiments were made to test the reproducibility of the disease characteristics. Two guinea-pigs were inoculated intraperitoneally with 0.25 ml. of a 24 hour broth culture. No visible disease process was evident in two weeks' time. At the end of that time, however, a purulent lesion at the site of inoculation of one of the animals was observed. The animal was killed. Blood agar culture of heart blood gave characteristic Group C hemolytic streptococci. No growth was obtained from other organs or lymph nodes. Culture of the pus from the purulent lesion showed that many hemolytic streptococci were present. On first isolation these hemolytic streptococci were mostly of the large mucoid type in which the capsules were easily demonstrable by staining with Anthony's and Wright's stains.

The streptococcus isolated from the pus was grown in Robinson's broth for 24 hours and used to inoculate six other guinea-pigs, each being given 0.25 ml. intraperitoneally.

The results were quite different from those of the preceding experiment. Within 24 hours a rapidly fatal septicemia was produced in 4 animals. Pure cultures of capsulated hemolytic streptococci were obtained from the heart blood, organs and peritoneum. The capsules were very easy to demonstrate, especially in smears taken from the peritoneum. In these smears the cocci were mostly in pairs and resembled pneumococci. The other two animals, although appearing ill during the first week, recovered with the characteristic formation of swollen lymph glands several weeks later.

Six mice were also inoculated intraperitoneally with the same first guinea-pig "passage" strain. Death occurred in 24 - 48 hours with an acute septicemia similar to that found in the guinea-pigs.

These experiments suggest that under certain circumstances this strain of streptococcus can acquire a rapid augmentation of virulence. Thus an organism, derived from animals in a phase of natural disease of increasing chronicity and diminishing morbidity, on primary passage produced a rapidly fatal disease similar to the most acute phase of the natural epizootic.

Conjunctival Inoculation

In the natural acute epizootic the first sign of infection was the inflammation of the conjunctiva. This inflammation preceded the appearance of enlargement of the lymph nodes by 1 - 3 weeks. This feature, although one of the most characteristic of the acute epizootic, was not found in similar epizootics reported by other workers.

Since it appeared that the organism might enter the animal by way of the conjunctiva, artifical infection was attempted by this route. The

righteyes of 6 guinea-pigs were swabbed with a broth culture of streptococci recently isolated from a guinea-pig lymph node. Six control animals were kept in a separate cage next to the inoculated group. The animals were inspected regularly for signs of conjunctivitis or lymphadenitis.

At no time was any eye lesion seen in any of the guinea-pigs. At the end of 2 weeks the lymph nodes were not enlarged, but by the end of 4 weeks all 6 animals swabbed with streptococci exhibited swollen lymph glands in the cervical region. Group C streptococci were recovered in pure culture from these glands. None of the control animals became infected.

These results indicate that infection introduced through the conjunctiva can induce the development of chronic cervical lymphadenitis indistinguishable from the natural disease.

This may well have an important bearing on the method of natural transmission. The habit of guinea-pigs of nuzzling one another makesit easy for streptococci to be conveyed directly from an open sore on the neck of one animal to the eye of another. The long period of latency, up to 4 weeks in some instances, between infection and the development of any detectable disease, also fits in very well with the epidemiology of the natural disease in its more chronic form. The failure of these experimental inoculations, however, to produce any evidence of acute eye inflammation suggests that some extra factor was present in the more acute epizootic.

Experiment on Aerial Transmission

Just above the cage containing the experimental herd and separated from it by a 1 inch wooden slat were kept 6 healthy guinea-pigs. These guinea-pigs remained healthy for 29 months of the investigation and showed no sign of

a streptococcal infection. These animals were always cared for before the infected herd in order to prevent the attendant from spreading the streptococci. Although the hemolytic streptococci would regularly be isolated from the straw and water of the lower infected group of animals, no indication of their spread to the upper group was found.

A similar experiment was conducted by Seastone (1939) except that his experiment allowed indirect transmission by excreta, bedding, etc., as well as by air. He was able for three months to prevent infection in healthy animals kept in the same cage as infected animals by separating the two groups by wire mesh grids 1 inch apart.

This shows that aerial transmission plays little part in the spread of natural infection at least in the phase of chronic endemicity. It is unfortunate that a similar experiment could not be carried out during the acute epizootic because there were certain indications that aerial transmission was occurring at that time. In particular the development of acute eye inflammation suggested the response to primary infection, yet this often occurred in animals kept in individual cages and separated in distance and time from any animals with open lesions.

BACTERIOLOGICAL INVESTIGATION

Morphological and Cultural Characteristics of the Epidemic Streptococcus

The streptococci were found in large numbers in the swollen lymph nodes of infected guinea-pigs. As might have been expected in these chronic lesions, Gram-negative as well as Gram-positive cocci were seen in smears. The organisms did not appear capsulated when stained with Anthony's capsule stain. In shape the cocci tended to be quite spherical

and uniform in size. Usually they appeared singly, in pairs or in very short chains.

When grown on blood agar plates the streptococcus grew aerobically, producing large, colourless, hemispherical colonies. Every culture showed a proportion of smaller but otherwise similar colonies. These tended to increase in number when subcultured repeatedly. The organisms from large and small colonies were identical in microscopic morphology. Subcultures of individual large or small colonies to blood agar plates produced a similar mixture of large and small colonies. Both types of colonies produced large clear zones of hemolysis in the surrounding medium.

On plain agar the streptococcus grew with difficulty forming minute, clear colonies. When inoculated into nutrient broth enriched with serum it produced a slightly granular growth with some sedimentation. However, in Robinson's glucose medium the streptococci produced a heavy turbid growth with no tendency to granulation.

In broth cultures the chain length increased to contain from 5 - 20 or more cocci.

Capsule formation was demonstrated with difficulty in broth cultures. It could be detected with Anthony's stain in 8 hour cultures but not in 24 hour cultures. Rapid intraperitoneal passage through 3 guinea-pigs produced large capsules which were easily stained by Anthony's or Wright's stains. In appearance the encapsulated organism resembled certain strains of capsulated pneumococci. The capsulated organisms were characteristically arranged in pairs with very little chain formation. Generally speaking the capsules surrounding the chains were less pronounced than the capsules surrounding the pairs of cocci.

When these capsulated organisms were grown on blood agar they tended to form larger mucoid colonies than the organisms isolated directly from the lymph node of the guinea-pig. However, this difference rapidly disappeared on further subculture.

Streptococcal Colony Variation in Cultures from Guinea-pig Lymphadenitis

The hemolytic streptococcus reported to be responsible for "lumps" among guinea-pigs seems to vary in its characteristics from epizootic to epizootic. Boxmeyer (1907) stated that an increase in the chain length of the organism in natural lesions tended to be associated with an increase in its virulence. He also reported that no capsule had been seen. Cunningham (1929) reported the tendency for the original large, viscid, mucilaginous colony to be replaced by a small, dry, granular type of colony upon repeated subcultures on blood agar. Seastone (1939) described the recovery from individual cases of a mixture of "chronic" and "virulent" strains out of which he was able to find 8 different variants classed according to colony formation, virulence, capsule formation and growth in broth. He found that the strain which was usually found associated with chronic guinea-pig lymphadenitis was an unstable, mucoid, capsulated organism exhibiting moderate pathogenicity. On blood agar plates two colony forms were invariably present. One was large and mucoid; the other small, smooth and more hemolytic. Repeated transfers of the mucoid strain produced pure mucoid strains but repeated transfers of the small colony strain did not produce a pure strain. Growth of the mucoid strain in broth gave both mucoid and non-mucoid strains on subculture to blood agar.

In the present investigation the organism associated with the chronic phase of the disease closely resembled the strain described by Seastone. On blood agar two different colony sizes were produced. Transference of either to a second blood agar plate produced a mixture of the two types of colonies. Repeated transfers of the two types, which was done daily for 3 weeks, failed to give rise to a single type of colony but always produced the two types. Thus it was not possible to reproduce the findings of Seastone who recovered a pure culture of the mucoid strain by this method. Since pure strains of the two distinct colonial types could not be isolated, it was not possible to compare their pathogenicity for the guinea-pig.

Colonies of streptococci associated with the acute phase of the disease tended to have a more mucoid appearance than those found with the chronic type of disease. This association of colonial morphology with pathogenicity was also noticed in the experimental production of disease.

Immunological Grouping of Streptococci

During this investigation of guinea-pig lymphadenitis it was necessary to check periodically the serological identity of the streptococci isolated from successive groups of animals. This necessitated a study of the grouping technique and its application.

The immunological grouping of streptococci is dependent upon the presence of a group specific carbohydrate substance "C" as shown by Lancefield (1928, 1933, and 1941). This "C" substance is closely connected to the streptococcal cell and anti-"C" precipitins are produced by animals inoculated with whole cells. When such antisera are covered by a layer of water-clear extracts of streptococci in capillary pipettes, a

precipitate forms at the junction of the antiserum and the extract of streptococci of the homologous group. Suitable extracts for grouping have been prepared by the following methods: -

1. Hot HCl extraction of centrifuged culture at pH 2.0 - 2.4, and subsequent neutralization with NaOH. This method was used by Lancefield.

2. Formamide extraction as done by Fuller (1938). This method is more laborious than Lancefield's method but yields a group specific antigen without confusing type fractions.

3. Enzyme extraction as carried out by Maxted (1948). The enzyme, obtained from cultures of Streptomyces albus, is mixed with a loopful of 18 hour culture to yield the "C" substance.

Lancefield's method (1933) of extraction of the group substance with hot HCl was tried but found to be tedious. Extracts of Streptomyces albus were then used. The simplicity of this method was stressed by Maxted (1948) and it was used for most of the tests.

The medium for the growth of S. albus was a modification of Maxted's as follows: -

Difco Yeast Extract	3 gm.
Fildes Peptic Digest	25 ml.
Na_2HPO_4	2 gm.
Dextrose	2 gm.
Agar	12.5 gm.
Phytone	5 gm.
Distilled water	1000 ml.

200 ml. of this medium in 12 oz. bottles was autoclaved for 20 minutes at 121°C and allowed to solidify as a slant covering one entire side of the bottle. A spore suspension of S. albus was then allowed to run over the surface of the agar.

Early attempts at production of the enzymic extract were unsuccessful. Good sporulation was apparently necessary for the production of the enzyme and this sporulation was found to occur at room temperature but not at 37°C, the temperature primarily used.

After the culture of S. albus had grown for about 4 - 6 days at room temperature the bottles were placed in the freezing compartment of the refrigerator and frozen overnight. The agar was thawed next day and the resulting mush was filtered through a Seitz sterilizing filter to yield a clear, light-brown fluid. This fluid was adjusted to pH 7.5 and stored in the refrigerator at 7°C until required.

The streptococcal grouping extract was prepared by adding a loopful of an 18 hour blood agar plate culture or a loopful of centrifuged broth culture to about 1 ml. of S. albus extract. The mixture was incubated for 3 hours at 37°C at which time the streptococci were lyzed to give a clear solution. Any remaining solid particles were removed by centrifugation. The supernatant was used to carry out the usual precipitin test.

Antisera of groups A to N were used. These were prepared by the Laboratory of Hygiene, Ottawa. For some of the tests, however, antisera to groups A and C were produced in the laboratory by inoculating rabbits with killed suspensions of the organisms.

The precipitin test was carried out in small Durham tubes. About 1/4 inch of grouping serum was introduced into the tube and a similar quantity of streptococcal extract was layered on the top. A

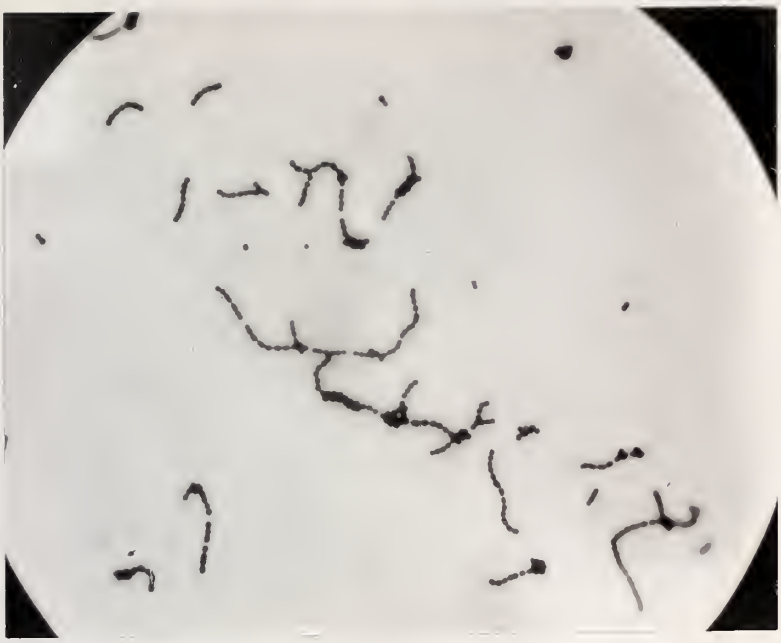
positive test was indicated in 10 - 20 minutes by a ring of precipitate at the junction of the two solutions.

Results

Most strains of streptococci isolated from the guinea-pigs were tested in this way and were found to belong to Group C. Usually Group A streptococci and Streptococcus viridans were used as controls.

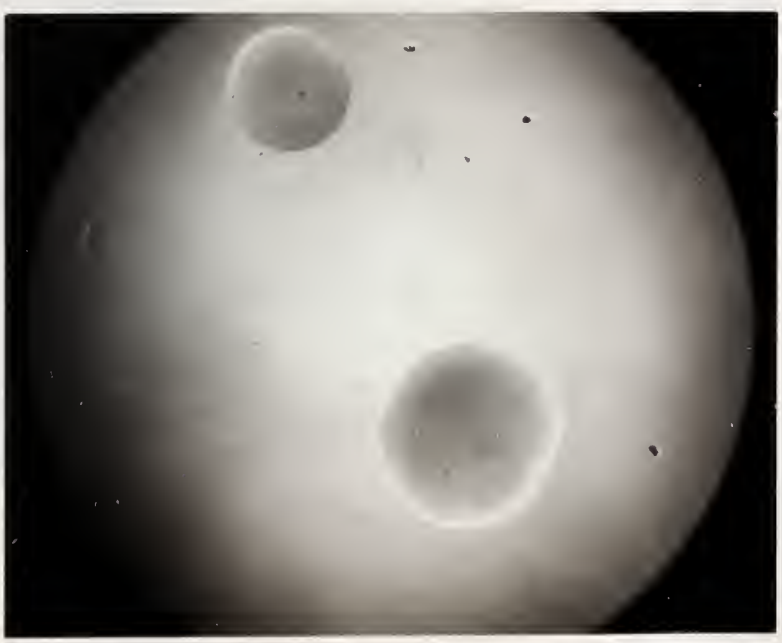
In some instances cross-precipitation occurred but the precipitation was never as strong as with Group C. The lysis of the streptococcal cells by S. albus extract was found to be a relatively simple procedure for the extraction of the carbohydrate "C" substance.

PLATE VIII



Gram stained smear of Streptococcus pyogenes epizooticus grown in Robinson's broth (X 1200)

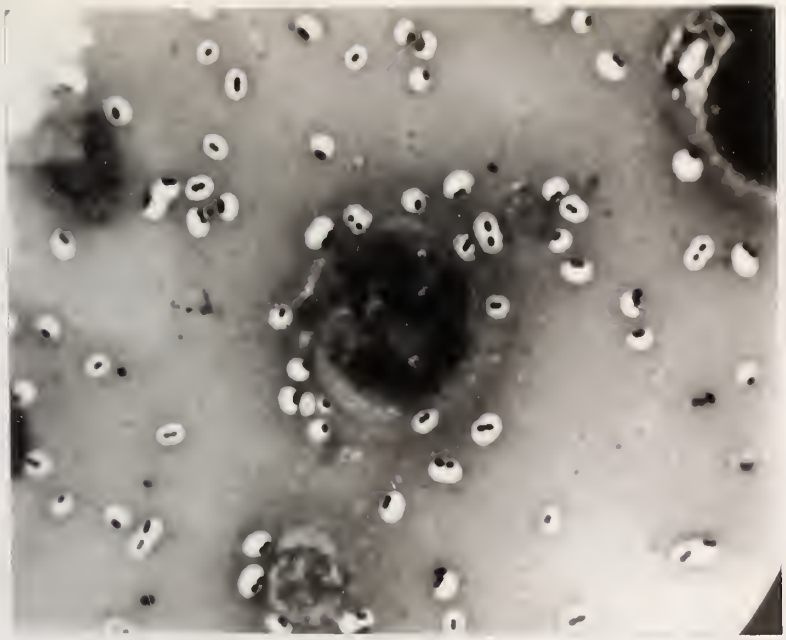
PLATE IX



Close-up of blood agar colonies of Streptococcus pyogenes epizooticus obtained from guinea-pig lymph node.

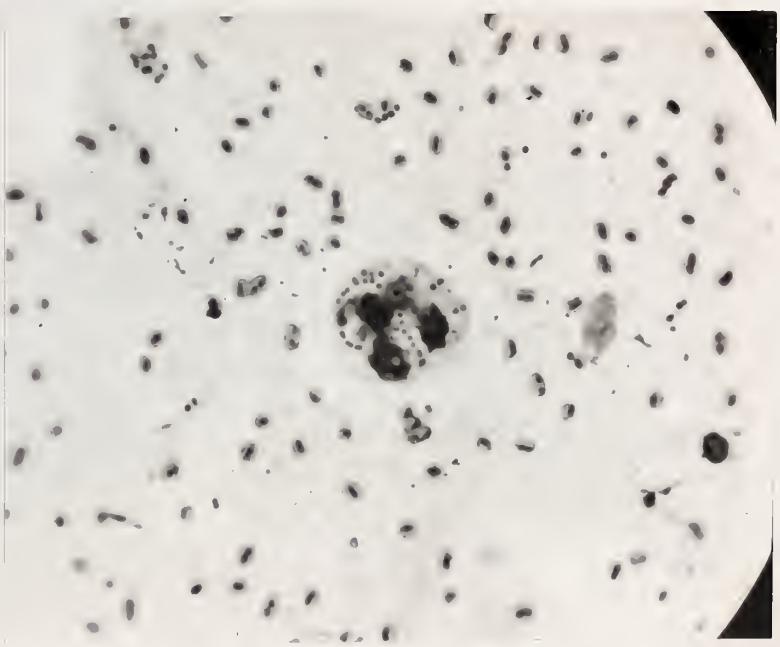
Note the two sizes of colonies. (X 16)

PLATE X



Gram stained smear of Streptococcus pyogenes epizooticus taken from peritoneum of guinea-pig (X 2000)

PLATE XI



Streptococcus pyogenes epizooticus taken from peritoneum of guinea-pig Stained with Anthony's capsule stain (X 1500)

HYPERSENSITIVITY TESTS

Moen (1936) described a skin test which he used successfully as a means of detecting possible carriers of the streptococci causing epizootic lymphadenitis in guinea-pigs. He suggested that such a skin test might be used as a means of obtaining guinea-pig breeding stock free of the streptococcus.

The test consisted of an intradermal injection of 0.1 ml. of streptococcal extract made by disrupting the organisms by drying and grinding in a ball mill. Infected animals were found to give a cutaneous reaction reaching its maximum in 24 hours and similar to a positive tuberculin reaction in appearance. Positive reactions were at least 10 mm. in diameter. Normal animals failed to react to this dose of bacterial extract.

At the Provincial Laboratory this skin testing method was used as one of the means to eliminate possible carriers of streptococci. The test was first tried on known infected animals, which were found to give good positive skin reactions.

Streptococci isolated in the current epizootic were grown in Robinson's glucose broth to obtain a heavy growth. The culture was centrifuged, washed several times in saline, and then concentrated to 1/20 of the original volume. It was then frozen and thawed about 8 times to kill and disrupt as many organisms as possible. Before use, the organisms in the suspension were all killed by placing in a water bath at 60°C, for 30 minutes. Sterility was checked by transferring loopfuls to blood agar and incubating at 37°C for 48 hours. Microscopic examination showed that the suspension contained many whole cells as well as disrupted cells.

The guinea-pigs were prepared for the test by shearing the hair of the abdomen with electric clippers. The inoculation was carried out with a 1 ml. syringe and a No. 22 needle. 0.1 ml. of the suspension was injected intradermally to produce a pronounced lump at the site of inoculation. The animals were examined at 24 hours and at 48 hours. If the area of reaction was larger than 10 mm. the test was considered to be positive. Maximum reactions were obtained within 24 hours and did not fade for several days. After the infected stock had been cleared out, selected samples of all fresh stocks introduced into the Provincial Laboratory were tested in this way and were found to be negative reactors. The subsequent finding that none of the animals ever developed streptococcal lymphadenitis confirmed the validity of the result.

The herd of naturally infected animals kept at the Department of Bacteriology was also tested for skin hyperreactivity. All these animals showed a positive reaction to the streptococcal suspension. Normal animals kept just above this group in the experiment on aerial transmission gave negative skin tests and remained free from disease.

Various guinea-pigs inoculated experimentally with the streptococci also developed positive reactions to the skin test antigen.

The results of these skin test experiments confirmed the findings of Moen (1936). Guinea-pigs infected with Group C streptococci of lymphadenitis showed an inflammatory skin reaction similar to a positive tuberculin test. This was found to be true of naturally infected animals as well as of artificially infected animals.

The value of this skin test in the detection of natural "carriers" could not be assessed completely because none of the healthy stocks

tested gave any reaction. In this instance the negative results were in accord with later clinical observations and bacteriological findings, because none of these animals developed streptococcal disease and the infection did not reappear subsequently in the Laboratory. The findings, therefore, suggest that skin-testing in the manner described will have a very definite place in the control measures in any future epizootic.

INVESTIGATION OF STREPTOLYSIN O PRODUCTION BY STREPTOCOCCI OF EPIZOOTIC LYMPHADENITIS

In the early part of this investigation the possibility was explored of applying the antistreptolysin test as a measure of control. The titration of antistreptolysin in the serum of human patients was known to be a dependable index of previous hemolytic streptococcal infection. If the same technique could be applied to animal infections it might indicate the animals which had experienced streptococcal infection and which might be possible carriers of the organism.

The Group C streptococci isolated from this epizootic lymphadenitis produced large, clear hemolytic zones surrounding the colonies on blood agar. The first necessity was to find out if the hemolysin was streptolysin O or streptolysin S. If it was streptolysin O, then presumably it would also be produced in the animal body and the specific antibody would be evolved in the course of infection.

Todd (1939) found that none of the few Group C streptococci he tested for streptolysin O production produced streptolysin O. He also showed that the hemolysins of Group C strains derived from human infections differ both chemically and serologically from the hemolysins of Group C

strains isolated from animal infections. Since he had tested only 2 Group C strains from guinea-pigs it was of interest in the present investigation to study the nature of streptolysin production.

This investigation necessitated an evaluation of procedures for streptolysin production and for the estimation of antistreptolysin levels in serum. In the current project these efforts were of little benefit, but they were applied to another investigation described in the second portion of this thesis. Detailed descriptions of materials and techniques have therefore been reserved for that section to which they are more pertinent.

The guinea-pig streptococcus when grown in Robinson's medium produced a potent hemolysin. This hemolysin, however, did not behave like streptolysin O produced by Group A strains. In storage it deteriorated rapidly at 7°C and could not be reactivated by reduction. The process was not due to irreversible oxidation because it occurred whether the streptolysin was kept under vaseline seal or not. Further, evidence was obtained that it was not naturally antigenic because neutralizing antibodies could never be demonstrated in the serum of infected guinea-pigs. In addition 4 rabbits inoculated intravenously with killed cultures and later with living cultures of the Group C streptococcus failed to develop antibodies which reacted in vitro with the streptolysin in its active state.

DISCUSSION

A remarkable feature of streptococcal lymphadenitis among guinea-pigs is the variation in the virulence of the disease. This has been evident in most published accounts and was well demonstrated in the current outbreak. Here an acute epizootic characterized by a high morbidity and a high mortality developed in a spontaneously infected herd but reverted to a chronic disease of low morbidity and negligible mortality in an experimental herd kept for further study. Although the urgency of the immediate control problem did not allow extensive bacteriological investigation during the acute episode it was determined that a disease process of similar acuteness could be produced experimentally in individual animals by a strain isolated during the chronic phase of the epizootic. Passage through one guinea-pig was sufficient to exalt the virulence of the organism to this extent. Thus it seems likely that the different characters of the natural disease may be dependent to a considerable extent on natural variations in the virulence of the infecting strain. The only bacteriological features which could be associated in any way with this variation were capsule formation, particularly evident in organisms observed in natural lesions in acutely ill animals, and a predominance of large colony variants in cultures from the same cases.

The epidemiology of the disease was studied most closely in the chronically affected experimental herd. Here it was established that infection was spread most likely by direct contact particularly with open lesions. The organism in this phase of its virulence did not spread by air or dust even to animals in the immediate vicinity of the infected herd.

All animals, however, introduced directly into this herd became infected. It seems doubtful if this limited method of transmission was entirely responsible for the spread of infection during the acute epizootic. Certainly the incubation period, or rather the period between infection and the development of clinical signs, was often of several weeks' duration in experimental animals. Thus animals could develop obvious disease long after they were isolated from the rest of the herd, but if the acute conjunctivitis, which was so noticeable in the acute epizootic, indicated a reaction to primary infection then infection was not always transmitted by direct contact.

The occurrence of eye lesions has not been reported by other workers and seems to have been a feature specific to the present epizootic. Its acute onset, its unilateral disposition, and the fact that in a proportion of cases it subsided only to be followed in a week or two by a typical cervical lymphadenitis, all suggested that it was a reaction to the primary invasion of a highly virulent organism. Although one was unable to reproduce the lesion experimentally it was possible to show conclusively that infection could occur through the conjunctiva, and that this infection could be followed after the usual incubation period by the characteristic lymphadenitis syndrome. If one accepts acute conjunctivitis as an index of primary infection then one must admit methods of transmission other than direct contact because this eye lesion developed in a number of animals in cage isolation from the rest of the herd. The most probable transmission in such cases was through air or dust. Experience with the experimental herd, however, indicated that this type of transmission dit not occur easily in the chronic phase of the disease, but did not rule out the possibility that the conjunctiva might be the most probable portal of organismal entry even in direct contact transmission.

The long period of latency of infection in experimentally and naturally infected animals explained the difficulty of control through observation and isolation of animals showing obvious lesions. At first it was thought that occasional animals which had developed infection might recover without showing any clinical evidence and that these animals might continue to carry the organism and act as potential reservoirs of infection within the herd. Two lines of investigation were developed in an attempt to detect such animals. The first was a skin test to demonstrate hypersensitivity to the infecting strain. This skin test was found to be positive in all animals which had developed lymphadenitis and to remain positive for at least a few weeks in animals in which the infected nodes had ruptured and discharged and the local lesion had healed. Thus the test should be of value in determining the presence of such infections in any group of animals and further evidence on the time of development and on the duration of this sensitivity should be obtained. In this work the negative skin tests in normal animals at least indicated the probable specificity of the test. The second line of investigation was on the development of serum antistreptolysins in infected animals. Useful as similar investigations may be in Group A streptococcal infections in humans, here they were of no value because of the failure of the Group C epizootic strain to produce antigenic streptolysin O.

SUMMARY

An outbreak of an acute streptococcal disease among guinea-pigs at the Provincial Laboratory is described. The disease was characterized by a conjunctival inflammation which subsequently led usually to the

development of either a rapidly fatal septicemia or a purulent lymphadenitis. Group C hemolytic streptococci were isolated from many animals and were considered to be the causative organisms.

This epizootic was eradicated by rigid control measures which included the removal of infected stock, thorough disinfection of the premises and the skin testing of healthy stock.

A naturally infected herd was maintained at the Department of Bacteriology for several years to study the chronic form of the disease. The number of animals was kept fairly constant and the epizootic was maintained by the addition of healthy guinea-pigs at various intervals. In this chronic form the disease was characterized by the formation of large lymph node swellings in the cervical region which would rupture to spread the contents containing the streptococci throughout the cage.

The organism associated with the chronic form of the disease was found to produce two sizes of colonies when grown on blood agar. This character was apparently intrinsic to the strain in question because it proved impossible to separate pure line strains giving either of the individual colony types. Its Group C nature was demonstrated repeatedly by the precipitin test. The organism was found to possess the ability to undergo spontaneous variation with readily demonstrable capsule formation in the acute disease producing strain.

Disease processes similar to those found in the natural infection were produced by artificial inoculation of the animals by both the conjunctival route and by the intraperitoneal route.

Skin testing of animals for streptococcal lymphadenitis may be a useful adjunct in keeping a guinea-pig stock free of streptococcal carriers. Positive reactors were found to be infected whereas the negative reactors were not infected. The use of Streptomyces albus extract for lyzing the

streptococci was convenient and simple.

Streptolysin O production by Group C streptococci of guinea-pig lymphadenitis was not demonstrable.

SECTION II

THE PREPARATION OF A STABLE ANTIGEN FOR THE ANTISTREPTOLYSIN O TEST AND A STUDY OF LOCAL ANTISTREPTOLYSIN O LEVELS

INTRODUCTION

Since 1932 when Todd first demonstrated the nature of streptolysin O, there has been a growing volume of evidence that a serum antibody to this hemolysin is regularly produced in human hemolytic streptococcal disease. Although a laboratory test such as the anti-streptolysin O test for the diagnosis of streptococcal disease has long been in demand, there have been several reasons why it has not gained rapid popularity for use as a diagnostic test.

At the beginning of this investigation the streptolysin O had to be produced in the laboratory and when the streptolysin O was standardized, the routine determination of antistreptolysin O content of sera was made available to physicians through the Provincial Laboratory.

As a subsequent study, since there appeared to be a lack of clinical evaluation of the antistreptolysin O test, a study of some of the clinical findings was conducted in an attempt to correlate these with the antistreptolysin O titre.

PART I

THE PRODUCTION, STANDARDIZATION AND PRESERVATION OF STREPTOLYSIN O

LITERATURE REVIEW

The Nature of Streptolysin O

The oxygen lability of the hemolytic activity of fresh broth filtrates of hemolytic streptococcus was first observed by Neill and Mallory (1926). They found that the addition of reducing agents re-activated the hemolytic capacity. Todd (1932) confirmed these findings and called the hemolysin streptolysin O because of its susceptibility to oxygen. Another hemolysin produced by Group A hemolytic streptococci in serum broth is known as streptolysin S.

Of the two hemolysins, streptolysin O has been found to be most important immunologically. It is highly antigenic both in animals and humans experimentally or naturally infected with Group A streptococci. Although the streptolysin is immunologically related to pneumolysin, tetanolysin and Clostridium welchii θ toxin (Todd, 1934), the antibody is readily and specifically identifiable.

Highly purified preparations of streptolysin O were obtained by Smythe and Harris (1940), Herbert and Todd (1941), and Bernheimer (1948). They showed that streptolysin O exhibits the properties and composition of proteins. It contains a relatively large amount of sulfur which occurs either in the disulfide or the sulfhydryl form, and is activated by substances which reduce disulfide links to the sulfhydryl groups.

When streptolysin is reduced from the -S-S- linkage by some reducing agents such as sodium hydrosulfite, cysteine or glutathione to form the -SH group it becomes hemolytically active. Hodge and Swift (1933) showed that streptolysin in the oxidized form could combine with anti-

streptolysin but did not have the capacity to hemolyze red cells. The hemolytic activity of reduced streptolysin can be specifically inactivated by antistreptolysin.

Herbert and Todd (1941) reported that the hemolytic activity was greatest at pH 6.5 and fell off markedly on both sides of the optimum pH. They also showed that at 0°C the activity of the hemolysin was 3%, and at 18°C 40%, of the activity at 38°C.

Streptolysin O Production

Todd (1932) found that he could produce streptolysin O using a broth containing horse meat infusion, peptone, and yeast extract. This medium was soon modified by Todd and Hewitt (1932) by substituting a phosphate buffer and certain growth stimulating substances such as glucose and sodium chloride for the yeast extract. Swift and Hodge (1933) used fresh beef heart fortified with glucose and 2% proteose peptone. Harmon and Feldman (1952) modified their medium by using proteose peptone, trypticase, phytone, glucose, and a buffer. Slade and Knox (1950) tested the effect of a number of reducing agents in attempting to produce the optimum amount of streptolysin. They were able to show that the production of streptolysin O was dependent upon the presence of reducing agents, whereas optimum growth occurred either in the presence or absence of such compounds. They found that cysteine-HCl in the very small optimum concentration of 0.0075% gave the highest production of lysin. Robinson, Crawford , and Roholt (1952) reported favourably on a medium containing this amount of cysteine-HCl.

The strain of streptococci used by most workers has been Richards' type 3 Group A streptococcus which is a regular producer of

large amounts of streptolysin O.

Rantz and Randall (1945) were able to purify the lysin partially by the addition of $(NH_4)_2SO_4$ to a concentration of 57%. The precipitate containing the streptolysin O was then dialyzed in cellophane sacs.

Streptolysin O Preservation

Todd (1932) showed that streptolysin treated with 0.1% sodium hydrosulfite could be preserved in the reduced state under vaseline seal in a refrigerator for long periods with no loss in potency. This method of storage has been the most popular. Rantz and Randall (1945) had a different approach. They stored the lysin in the oxidized state and reduced it to the active state just before use.

The commercial Streptolysin O Reagent comes in a desiccated form and has the necessary amount of reducing agent in it. It is reactivated just before use by the addition of distilled water.

Streptolysin O and Antistreptolysin O Standardization

Standardization of the lysin was originally done according to its hemolytic capacity. Todd (1932) arbitrarily standardized the streptolysin O unit as that quantity of reduced streptolysin which when diluted to 0.5 ml. with normal saline just completely hemolyzes 0.5 ml. of a 5% rabbit red cell suspension at 37°C in 1 hour. He then defined one unit of antistreptolysin O as that amount of serum which just neutralizes $2\frac{1}{2}$ minimum hemolytic doses (M.H.D.) of streptolysin O. The antistreptolysin O titre was at first recorded as the volume of serum required to neutralize $2\frac{1}{2}$ M.H.D.'s of hemolysin but later the method was modified by recording the reciprocals of the fractions and describing the resulting whole

numbers as units of antistreptolysin.

Todd (1932) showed that both reduced and oxidized streptolysin were active antigens in vivo. In vitro he found that both the reduced and oxidized streptolysin combined with antistreptolysin O. Hodge and Swift (1933) showed that it was more important to know the combining capacity of the streptolysin rather than the exact hemolytic power in terms of minimum hemolytic dosage. In the first stage of an antibody titration the streptolysin combined with the antibody, apparently in multiple proportions; in the second stage the lysis of erythrocytes is the indicator for the presence of uncombined streptolysin. The first can take place in either the reduced or oxidized form of streptolysin. The second, however, can take place only in the reduced form of streptolysin. Thus they argued that the combining capacity of streptolysin could not be estimated from its strength and therefore a new standard based on combining capacity should be adopted. The combining capacity was found to remain at a constant level provided the streptolysin was kept cold and sealed either in the reduced form, the oxidized form or a combination of the two.

The standard combining power, one combining unit, was designated as that amount of streptolysin which was completely neutralized in 1 ml. of immune serum diluted to contain 1 unit of antistreptolysin O. A unit of antistreptolysin was based on the original Todd serum to which he gave an antistreptolysin O value of 20,000 units per ml. It appears that most laboratories have based the antistreptolysin O titres on these standards. In the commercial preparation of streptolysin O, Difco Laboratories have used these same standards.

MATERIALS AND METHODS

PREPARATION OF STREPTOLYSIN O

Medium

Several different media were used in the production of streptolysin O. After some experimentation the medium reported by Robinson, Crawford and Roholt (1952) was found to be superior in the production of streptolysin O.

Fresh lean beef was finely ground, mixed in the proportion of 1 pound to 1000 ml. of distilled water, and left in the refrigerator overnight. The infusion mixture was strained through several thicknesses of gauze into a 2 litre Erlenmeyer flask. To each litre of filtrate was added: -

20 gm. neopeptone or Bacto proteose peptone No. 3.

30 ml. of 1 N NaOH.

The mixture was boiled for ½ hour and then cooled in cold water. The broth was filtered through ordinary Whatman filter paper No. 3 and sterilized in the autoclave at 15 lbs. for 20 minutes.

A buffer solution sterilized by Seitz filtration was added to the medium. This buffer was prepared as follows: -

Dextrose	12.0 gm.
$NaHCO_3$	8.0 gm.
NaCl	8.0 gm.
$Na_2HPO_4.12H_2O$	8.0 gm.
Distilled water	200 ml.

50 ml. of this buffer was added per litre of medium at the time of inoculation. Also at the time of inoculation 25 ml. of the following freshly prepared solution was added per litre of medium: -

1 N NaOH	2.0 ml.
Saline	98 ml.
Cysteine-HCl	0.3 gm.

This solution was checked for pH of 6.5-7.5, sterilized by filtration and used immediately. This gave a cysteine concentration of 0.0075% found to be optimum by Slade and Knox (1950).

Inoculum

The strain of streptococci used was Richards' Group A type 3 strain. This strain was previously grown in the same broth and transferred several times to insure a rapidly growing culture. The medium was inoculated with 1% of its own volume of a 6 hour culture, the flask plugged with a cotton stopper, and incubated for 16 hours at 37°C. At the time of inoculation purity of the culture was checked by microscopic examination and blood agar culture.

METHOD OF HARVESTING THE STREPTOLYSIN O

After incubation sodium hydrosulfite was added to a concentration of 0.1%, the flask evacuated by a vacuum pump to remove the dissolved air and placed in the refrigerator for several hours to cool. The cooled culture was centrifuged in 50 ml. tubes at 3000 r.p.m. for 30 minutes. The reduced liquid streptolysin O was siphoned off into 1 oz. H-53 screw-capped bottles covered with $\frac{1}{2}$ inch of vaseline seal. This lysin was stored for 6 weeks to insure stability before use.

STANDARDIZATION OF STREPTOLYSIN O AND ANTISTREPTOLYSIN O

Several samples of sera were obtained from Dr. L.A. Rantz. These had been standardized against Todd's original serum containing 20,000 units per ml. The sera were as follows: -

Taccino	166 units/ml.
D. Smith	125 units/ml.
M.S. 187	250 units/ml.

The following standards were also received from Dr. Bynoe, Laboratory of Hygiene, Ottawa: -

Gamma globulin	1000 units/ml.
Pooled antiserum A	1250 units/ml.
Pooled antiserum B	200 units/ml.
Pooled antiserum C	50 units/ml.

Laboratory prepared batches of streptolysin O were titrated against the above standards of antistreptolysin O. The method used was essentially that of Hodge and Swift (1933) as adopted by Rantz and Randall (1945). The combining unit is the largest quantity of streptolysin which causes no hemolysis in the presence of a standard amount of antistreptolysin. This was determined by titrating dilutions of streptolysin of unknown strength against a constant volume of standard serum diluted with saline so that 1 ml. contained 1 unit of antistreptolysin O. The method is given in table form below.

TABLE III

Method of Determining the Combining Unit of Streptolysin O

Reagent	Volumes in ml.									
Streptolysin	0.5	0.45	0.40	0.35	0.30	0.25	0.20	0.15	0.10	0.05
Saline	---	0.05	0.10	0.15	0.20	0.25	0.30	0.35	0.40	0.45
Standard anti-streptolysin 1 unit	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Incubate 15 minutes before adding cells										
5% R.B.C.'s	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5

The tubes were incubated for 45 minutes after the addition of the cells and checked for hemolysis by centrifuging. The slightest degree of hemolysis showed the presence of some free hemolysin. Therefore the first tube showing complete absence of hemolysis indicated the largest volume of streptolysin completely neutralized by 1 unit of antistreptolysin. This volume contained 1 combining unit.

Storage of Streptolysin O

All streptolysin O prepared was stored at 7°C except where stated otherwise.

Freeze drying of Streptolysin O, Antistreptolysin O and Streptococci

Sera containing antistreptolysin were freeze dried to be used as standards for the titration of streptolysin O. 1.0 ml. was pipetted into 3 ml. freeze drying tubes. For use these vials were broken and made up to volume with distilled water just before use.

Streptolysin O was first standardized and placed in 25 ml. tubes and freeze dried. Just before use the tubes were broken and rehydrated with distilled water to contain 1 combining unit in 0.5 ml. of lysin.

Streptococci were freeze dried in 1 ml. ampoules, 0.2 ml. of a 24 hour broth culture was placed in each tube.

The freeze drying was done in an Edwards' Centrifugal Freeze Drier, with phosphorus pentoxide as the desiccant. The primary drying was done in the centrifugal unit while the secondary drying was done by attaching the tubes to the nipples of an exhaust manifold. After drying the tubes were sealed under vacuum with a gas flame.

All freeze dried material was kept in the refrigerator at 7°C.

EXPERIMENTAL RESULTS

THE PRODUCTION OF STREPTOLYSIN O

An attempt was made early in the investigation to find a good streptolysin O producer among hemolytic streptococci isolated at the Provincial Laboratory. Stuart (1936) found that very considerable variations were encountered in various strains of streptococci and therefore not all strains could be used for the production of streptolysin O.

The organisms were picked from blood agar into Harmon and Feldman's broth, incubated for 24 hours, centrifuged and tested for hemolytic potency. Results are shown in Table IV.

TABLE IV

Production of Streptolysin O by Various Strains of Streptococci

Strain No.	Supernatant in ml.					
	0.5	0.4	0.3	0.2	0.1	0.05
1	C	C	+	+	0	0
2	+	+	+	±	0	0
3	+	+	+		±	0
4	+	+	+	±	0	0
5	+	+	±	0	0	0
6	+	+	+	±	0	0
7	+	+	+	+	±	0
8	+	+	±	0	0	0
9	+	+	+	±	0	0
10	C	C	+	+	+	±
11	+	+	+	±	0	0
12	+	+	+	+	±	0
13	+	+	+	+	±	0
14	+	+	±	0	0	0
15	+	+	±	0	0	0
16	+	±	0	0	0	0
Richards'	C	C	C	C	+	+

C = complete hemolysis
\+ = partial hemolysis
± = trace hemolysis
0 = no hemolysis

Strain number 10 was found to be the most hemolytically potent strain. For preliminary production of streptolysin this strain was used until Richards' type 3 strain became available, which was found to be a better producer of streptolysin than strain No. 10.

Streptolysin Production and Culture Medium

Streptococcus strain No. 1 was used to test the difference between the medium of Robinson (1952) and that of Harmon and Feldman (1952). Robinson's medium for this experiment did not contain cysteine-HCl. The cultures were centrifuged and tested for hemolytic potency immediately following incubation with no further reduction with sodium hydrosulfite.

TABLE V

Streptolysin O Production and Culture Medium

Medium	Supernatant in ml.					
	0.5	0.4	0.3	0.2	0.1	0.05
Robinson	C	C	C	+	+	+
Harmon	C	C	+	+	0	0

Robinson's medium was found to be superior in streptolysin O production. Experiments with some of the other strains gave similar results. The greater production of streptolysin in Robinson's medium was probably due to enrichment with glucose.

In order to assess the value of proteose peptone No. 3 and neopeptone (Robinson recommends either one) several batches of medium were prepared containing (a) no added peptone, (b) 20 gm. proteose peptone No. 3, (c) 20 gm. neopeptone, and (d) 20 gm. proteose peptone No. 3 and 20 gm. neopeptone. No significant difference was observed in the

amount of streptolysin production in any of these groups except in (a) where the lack of peptone was reflected in diminished culture density as well as diminished streptolysin O production.

Streptolysin O Production and Reducing Agents

The effect of the lack of sodium hydrosulfite as a reducing agent for the testing of fresh cultures of hemolytic streptococci was investigated. Sodium hydrosulfite solution was added to the supernatants to make a final concentration of 0.1%.

TABLE VI

Streptolysin O Production and Reducing Agent

Sodium hydrosulfite	Strain No.	Supernatant in ml.						
		0.5	0.4	0.3	0.2	0.1	0.05	Control
0.1%	1	C	C	C	+	+	+	0
0.1%	10	C	C	C	+	+	+	0
---	1	+	+	+	+	+	±	0
---	10	+	+	+	+	+	±	0

These results indicate the value of a reducing agent such as sodium hydrosulfite in increasing the hemolytic action of streptolysin O.

PRESERVATION OF STREPTOLYSIN O

Preservation of streptolysin O was investigated at refrigerator (7°C) temperature. Emphasis of the experiment was placed on the combining potency of the streptolysin rather than on the hemolytic capacity. The following table gives values of streptolysin in ml. of filtrate equivalent to 1 combining unit.

TABLE VII

Streptolysin O Preservation at 7°C

Batch No.	1st test		2nd test		3rd test	
	Age months	C.V.*	Age months	C.V.	Age months	C.V.
1	1	0.10	4	0.10		
2	1	0.10	7	0.10	14	0.10
3	1	0.05	3	0.05	7	0.05
4	1	0.10	5	0.10		
5	1	0.05	5	0.05		

* C.V. = ml. of streptolysin equivalent to 1 combining unit.

These results show that standardized streptolysin when stored at refrigerator temperature with 0.1% sodium hydrosulfite and a vaseline seal may keep for as long as 14 months without deterioration of the combining unit.

Effect of Further Addition of Sodium Hydrosulfite in Stored Streptolysin as Shown by the Combining Test

It was of interest to know if reduction with 0.1% sodium hydrosulfite was sufficient to keep the streptolysin O in a completely reduced state when stored under a vaseline seal for long periods. Seven different batches were tested to note if further reduction with 0.1% sodium hydrosulfite would alter the results of the combining unit. Streptolysins No. 5a, 5b, 5c and 5d were of the same batch, but 5b was in a smaller container, while 5c was kept at -6°C and 5d at - 22°C.

TABLE VIII

Effect of Further Addition of Sodium Hydrosulfite to Stored Streptolysin O

Batch No.	Age in months	Combining unit	
		No $Na_2S_2O_4$	1% $Na_2S_2O_4$
4	14	0.10	0.10
5a	7	0.05	0.05
5b	7	0.05	0.05
5c	7	0.30	0.35
5d	7	0.10	0.10
6	5	0.10	0.10
7	5	0.05	0.05

Since the antistreptolysin O test is dependent upon the hemolytic nature of uncombined, reduced streptolysin, it is important to have the streptolysin in a fully reduced state at the time of use. These results showed no change in the hemolytic nature of the streptolysin with further addition of sodium hydrosulfite when tested in the form of a combining test. Thus, for all practical purposes of antistreptolysin titrations, the streptolysin was kept in an adequately reduced state during storage.

Storage of Streptolysin O at Varying Temperatures

Streptolysin O was stored at 4 different temperatures, for a period of 7 months. The temperatures were: room temperature, 7°C, -6°C, and -22°C. The streptolysin was kept in a reduced state under a vaseline seal. The results are expressed as the amount of streptolysin required to neutralize 1 unit of antistreptolysin. The original streptolysin when

standardized required 0.05 ml. to be neutralized by 1 unit of antistreptolysin O.

TABLE IX

Storage of Streptolysin O at Varying Temperatures

No.	Temp.	Streptolysin in ml.
1	room	0.10
2	7°C	0.05
3	-6°C	0.30
4	-22°C	0.10

Temperatures below freezing were found to promote deterioration of the streptolysin O. It was noticed that often temperatures were not low enough to produce quick and complete freezing of the streptolysin-containing broth which seemed to separate into an upper frozen layer and a lower concentrated broth layer only partially frozen. At 7°C the streptolysin O potency remained the same while at room temperature there was a slight amount of deterioration of the streptolysin O.

Storage of Streptolysin O in Diluted Form

Standardized streptolysin O was diluted to contain 2 combining units per ml. and stored under a vaseline seal in screw-capped 1 oz. bottles at room temperature and at refrigerator temperature (7°C) for 3 months.

A serum containing 125 units/ml. was used. The test method was the same as the regular antistreptolysin test.

TABLE X

Storage of Diluted Streptolysin O

Streptolysin	Storage	A.S.T.
diluted 2 u/ml.	room temp.	166, 250
	7°C	125, 125
not diluted	7°C	125, 125

Streptolysin O stored in the diluted form at room temperature showed a slight decrease in combining capacity as indicated by an increased A.S.T. Streptolysin stored at 7°C, however, maintained the same combining capacity as undiluted streptolysin stored in the refrigerator. These results show that streptolysin can be stored in the refrigerator in the diluted form for 3 months with no loss in combining capacity if sealed with vaseline to exclude atmospheric oxygen.

Storage of Streptolysin O in the Freeze Dried State

Several batches of streptolysin O were freeze dried in 25 ml. ampoules in the Edwards' Centrifugal Freeze Drier and stored at 7°C.

The streptolysin was found to keep its original potency for as long as 7 months by this method. Although this is the ideal way to store the streptolysin only 12 ampoules were handled at a time by the machine in one day and it thus proved to be a laborious method.

PLATE XII

Edwards' Centrifugal Freeze Drier

DISCUSSION

The availability of a high quality stable preparation of streptolysin O is essential to any laboratory offering antistreptolysin tests as a service. At the beginning of this investigation no such preparation was commercially available and information about the best method of preparing and storing the material was scattered and inconclusive. The preceding experimental work has shown that the production and maintenance of preparations of streptolysin O is a comparatively simple matter and well within the capacity of the average bacteriological laboratory. The points which require most attention are the selection of a suitable strain, the use of the best culture medium and certain simple precautions in storage of the final preparation.

Richards' Group A type 3 strain has been found superior to the other strains in the production of streptolysin. The medium reported by Robinson, Crawford and Roholt (1952), in which 0.0075% cysteine-HCl is incorporated, was found superior to the other media tested.

The preservation of streptolysin in a stable form with no reduction in potency for months at a time is a necessary requirement for accurate routine titration of antistreptolysin. That this can be done has been demonstrated by keeping the hemolysin reduced with 0.1% sodium hydrosulfite and covered with a ½ inch layer of vaseline and a screw-cap cover. Once a tube is opened it should not be kept for any length of time due to oxidation of the streptolysin to the non-hemolytic form. Storage temperature appears to be important in preserving the streptolysin. At room temperature it deteriorates rapidly especially if left uncovered. The streptolysin also appears to deteriorate when kept at temperatures just below freezing.

During this investigation a commercial preparation in dehydrated form was put on the market by Difco. This material has been tested with our own laboratory preparations and titrated against standard antisera obtained from a variety of sources. The product seems to be of excellent quality and stability and can be recommended. The cost, however, is relatively high and many laboratories, certainly those carrying out large numbers of antistreptolysin tests, may prefer to produce their own antigen. In such instances the Difco material still remains as an excellent reference, both for occasional confirmatory readings and for a ready source of standard material.

SUMMARY

(1) Although some good streptolysin O producing strains of streptococci were found, none were as good as Richards' Group A hemolytic streptococci. This strain has proven to be a good stable streptolysin O producer.

(2) The medium reported by Robinson has been found superior to the other media for the production of streptolysin O. The concentration of 0.0075% of cysteine in the medium was found to give a high yield of streptolysin O.

(3) The preservation of streptolysin O at refrigerator temperatures when reduced with sodium hydrosulfite and kept in vaseline sealed bottles has been shown to result in no drop in potency of the streptolysin O for as long as 14 months.

(4) Streptolysin O diluted to contain 2 units per ml., sealed as above and stored at 7°C for 3 months, showed no demonstrable drop in combining power.

(5) The storage of streptolysin at temperatures somewhat below freezing and also at room temperature has been shown to result in a drop in potency of the streptolysin.

PART II

ANTISTREPTOLYSIN O LEVELS IN HEALTH AND IN DISEASE

INTRODUCTION

The determination of serum antistreptolysin titres is increasingly being requested to assist in the diagnosis of certain types of hemolytic streptococcal infection. Particularly in diseases such as rheumatic fever and its clinical sequelae, or in glomerulo-nephritis the streptococcal aetiology may be suspected but cannot be proved by direct bacteriological investigation. Yet if the infection is streptococcal, effective treatment and prophylaxis may be available in penicillin. The indirect but convincing evidence of high or rising antistreptolysin titres in a patient's serum may lead to specific diagnosis and specific treatment.

This investigation has been undertaken to confirm the significance of antistreptolysin determinations in certain diseases and also to determine the average level of the antibodies in healthy persons of various age groups in the Edmonton area. This last information is essential to the assessment of the significance of various titres as they may be found in individual patients.

LITERATURE REVIEW

Ever since Todd (1932) demonstrated that streptolysin O was antigenic to horses and to human beings many investigators have tried to assess the value of antistreptolysin O titration in the diagnosis of various streptococcal diseases. According to Swift (1947) at least 90% of patients infected with hemolytic streptococci developed demonstrable amounts of antistreptolysin which might persist for months and possibly years. Because of this persistence its value as an index of recent streptococcal infection is open to question. Stuart (1936) who studied a group of patients mostly suffering from tonsillitis, scarlet fever or puerperal infections found that the severity of the streptococcal disease had no relationship to the rate and height of antibody response which appeared to depend more on the individual case. Swift (1947), however, found that in rheumatic fever the elevations were much more constant than in the other streptococcal diseases.

The antistreptolysin O content of normal sera varies over a relatively wide range. Todd (1938) found a mean value of 83 units for normal persons with the following distributions: (1) 75% had titres less than 100 units; (2) 20% had titres from 100-200 units; and (3) 5% had titres in excess of 200 units. Longcope (1936) showed that 50 units was the usual normal level for adults in a southern United States city. Rantz, DiCaprio and Randall (1952) found mean titres of 150 units for healthy subjects in the 5-7 year age group, 184 for 8-12 year age group, 102 for medical students, 46 for 40-49 age group and 48 for 50-59 age group.

In active rheumatic fever Rantz and his co-workers found that the titre was 166 units or more. In children they found a mean of 398

units and in adults they found a mean of 553 units. In glomerulo-nephritis cases the antistreptolysin titres were 250 units or more with a mean of 887 units per ml. In cases with a nephrotic syndrome they invariably found excessively low concentrations of antistreptolysin O with only 1 case having more than 12 units per ml.

Rantz, Maroney and DiCaprio (1951) suggested that there were three fairly well defined patterns of antistreptolysin O response following Group A hemolytic streptococcal infection in children. The first pattern was the lack of response found in children about 1 year old. This was demonstrated in children suffering from respiratory infections complicated by streptococcal disorders such as otitis media, purulent rhinitis and pyoderma. The second pattern was found in a group whose average age was 3 years. Here the mean peak titre following streptococcal disease was 235 units but the antistreptolysin rapidly declined to low levels. The third pattern was found in children whose average age was 5 years. In this group there was a still more vigorous antibody response with levels from 100 to 2,500 units (mean 495 units) and a much more gradual decline in titre. Lippard and Johnson (1935) found that normal infants less than 17 months averaged 24 units per ml. while older infants averaged 66 units.

Hollinger (1953) could not detect any absolute or specific difference in the antistreptolysin levels between normal and rheumatic children. A high titre (over 250 units) was certainly related to streptococcal disease but had no specific significance in relation to rheumatic fever. On the other hand he found that a low level (50 or less) obtained repeatedly in the same child was highly reliable in confirming the absence of active rheumatic fever.

MATERIALS AND METHODS

THE TECHNIQUE OF TITRATING THE ANTISTREPTOLYSIN O CONTENT OF SERA

The antistreptolysin O test consisted of adding a constant volume of streptolysin O containing 1 combining unit to various dilutions of the test serum, incubating for 15 minutes, then adding a constant volume of a 5% rabbit red cell suspension, reincubating and centrifuging. The antistreptolysin O titre was the reciprocal of the highest dilution showing no sign of hemolysis. The technique is essentially that described by Rantz and Randall (1945).

1. Method of Obtaining Sera

Blood was drawn aseptically into regular blood tubes in which it was allowed to clot. The serum was poured off and centrifuged to remove any red cells.

2. Preparation of Rabbit Red Cell Suspension

A 5% suspension of thrice washed rabbit red cells was used as the indicator of uncombined streptolysin. Rabbit blood was obtained from the jugular vein by pricking it with a sharp No. 16 needle which was quickly withdrawn. The blood was allowed to drip into a tube containing isotonic oxalate as an anticoagulant. The tubes for receiving the blood were prepared as follows: -

Ammonium oxalate	1.2 gm.
Potassium oxalate	0.8 gm.
Distilled water	100 ml.

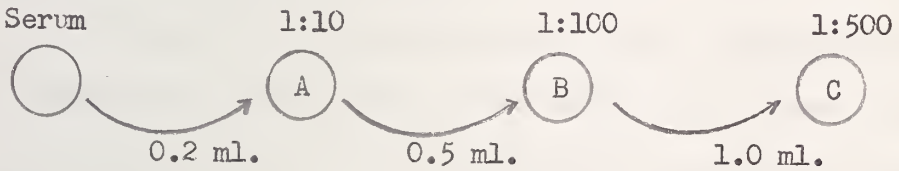
0.5 ml. of this solution was transferred to each bottle and allowed to dry at room temperature. After the water had all evaporated the bottles were covered and stored. In use 5 ml. of blood was placed in each tube.

Cells obtained in this way and made up to a 5% suspension with

saline were found to keep up to 2 weeks with no hemolysis.

3. Serum Dilutions

Although sera could be inactivated at 56°C for 30 minutes to destroy the complement, this procedure was found unnecessary. The method of Rantz and Randall (1945) required 0.5 ml. of serum. For this investigation only 0.2 ml. of serum was used. The serum dilutions were made up in two stages. In the first stage 1:10, 1:100 and 1:500 dilutions were made with buffer saline in 13 x 100 mm. tubes.



Saline 1.8 ml. 4.5 ml. 4.0 ml.

Final dilutions were made up according to the following table: -

TABLE XI

Serum Dilutions

	A 1:10		B 1:100					C 1:500					Cell Control	Lysin Control
Tube number	1	2	3	4	5	6	7	8	9	10	11	12	13	14
ml. diluted serum	0.8	0.2	1.0	0.8	0.6	0.4	0.3	1.0	0.8	0.6	0.4	0.2	---	---
ml. buffer saline	0.2	0.8	0.0	0.2	0.4	0.6	0.7	0.0	0.2	0.4	0.6	0.8	1.5	1.0
units	12	50	100	125	166	250	333	500	625	833	1250	2500		

Normally only the first 10 tubes were used but if higher titres were expected the last two tubes could be included. Cell controls and lysin controls were not necessary in doing batches of tests because un-

affected tubes acted as controls.

The buffer saline for diluting the streptolysin O and making the volumes constant in the tubes was prepared as follows: -

NaCl	4.2	gm.
KH_2PO_4	3.17	gm.
Na_2HPO_4	1.81	gm.
H_2O (distilled)	1000	ml.

4. Streptolysin O Dilution

Streptolysin O titrated against Todd's standards was used. This was diluted with isotonic buffer just before use. Streptolysin O which had been diluted for more than 2 hours was not used since its potency might have decreased due to inactivation with atmospheric oxygen.

After incubation the tubes near the end point were centrifuged to read for hemolysis. The first tube in the series in which no hemolysis was recorded indicated the presence of 1 combining unit of antistreptolysin. The titre of antistreptolysin in the original serum was read as the reciprocal of the serum dilution in that tube.

5. Control Sera

Sera of known antibody content were used in order to ensure the accurary of the test especially when new batches of any reagents were used. Freeze dried sera were found to be quite adequate as controls.

CASE STUDIES

Blood samples from patients in several local hospitals were obtained through the Provincial Laboratory. These specimens were submitted for various routine tests but a portion of the serum was received

by the Department of Bacteriology for antistreptolysin titration. The antistreptolysin O test was performed as described in the previous part of this section. A record of the laboratory number, patient's name, physician's name, and hospital number was kept so that the patient's history and diagnosis could be obtained later.

With the assistance of the University Hospital a survey was conducted to determine the antistreptolysin O titres of mothers and infants.

Several months after the antistreptolysin O test was made available to physicians, mimeographed sheets (page 79) were distributed describing the possible value of the antistreptolysin O test and the titre which might be expected.

The Antistreptolysin O Test

The presence of antistreptolysin O in human serum is evidence of Group A haemolytic streptococcal infection. Many workers have shown that high titres of such antibodies can be correlated with recent infection with haemolytic streptococci. Accordingly the demonstration of high titres of antistreptolysin O may be of importance in the diagnosis and prognosis of rheumatic fever. In this disease and also in streptococcal glomerulonephritis high concentrations of antistreptolysin O may be expected. Most people, however, have suffered from haemolytic streptococcal infection at some time in their lives and therefore possess antistreptolysins in their blood serum. The physician will be interested in any information which helps to differentiate such "normal" antibody levels from those more indicative of recent infection.

The antistreptolysin titre for normal infants is low -- 12 units or less. For normal children the titre may vary depending on whether they have been exposed to a streptococcal infection. Usually it runs from 12 to 100 units. In adults the titre usually ranges from 50 to 125 units. An antistreptolysin O titre of 200 units or more can be considered as suggestive of recent Group A streptococcal infection. Low titres such as 50 units or less give strong evidence against the presence of either rheumatic fever or glomerulonephritis, and high titres, such as 300 units or more, are indicative of a recent streptococcal infection. In cases of acute rheumatic fever the titre is frequently from 250 units to 500 u/cc., but it must always be remembered that a high reading of even this order is never absolutely diagnostic of rheumatic fever though a very low reading may practically exclude it.

The limitations of single antistreptolysin determinations must be stressed, but a rising titre between two tests always indicates current streptoooccal activity. Usually specimens are examined at two week intervals. Any rise in the level of antistreptolysin titre is significant, whether it is from 12 to 125 units, or from 125 to 300 units. An increase in titre or a drop in titre may indicate the stage of illness.

The administration of penicillin or salicylates has little effect on the antistreptolysin O titre, but the administration of cortisone or ACTH may lower the level of antistreptolysin O in the serum.

RESULTS

RESULTS

During the year 1954 over 200 diagnostic antistreptolysin O determinations were made, most of these during the latter half of the year when the availability of the test became more widely known. The case histories of 116 patients were available for investigation. From these records the patients could be divided into four groups.

1. The first group includes all cases of rheumatic fever, confirmed or tentative, and of chorea. This group is called the rheumatic fever group.

2. The second group contains cases of glomerulonephritis. Included in this group are cases diagnosed as nephritis, acute glomerulonephritis, chronic glomerulonephritis and acute hemorrhagic nephritis. This group is referred to as the glomerulonephritis group.

3. The third group consists of patients who suffered from various other diseases in which streptococci are the usual aetiological agents. These include such diseases as otitis media, tonsillitis, pharyngitis, scarlet fever, etc. This group will be referred to as the streptococcal disease group.

4. The fourth group consists of patients who suffered from diseases other than streptococcal disease.

Groups 1, 3 and 4 have each been divided into 4 age groups in an attempt to assess antistreptolysin variations in the various age groups. The figures for the mean antistreptolysin O titre of each age group are given to show the spread or range of the determinations.

Group I. Rheumatic Fever.

In a total of 39 cases investigated the mean antistreptolysin O titre was found to be 519 units per ml. There was no significant difference in the findings in different age groups. Although the mean titre was quite high, titres varied from 50 units to 2500 units. Two cases of Sydenham's chorea were in this group with antistreptolysin O titres of 333 and 833 units.

Of the 35 cases showing titres of 166 or above, 32 were diagnosed clinically as definite rheumatic fever; the remaining cases being left with the equivocal diagnosis of "possible rheumatic fever" or "rheumatic fever or rheumatoid arthritis." The 4 cases with lower titres were diagnosed as follows: 1. Erythema nodosum with a differential diagnosis of rheumatic fever (A.S.T. 125).

2. Acute rheumatic fever (A.S.T. 50).

3. Subacute rheumatic fever. Age 77. (A.S.T. 50).

4. Rheumatic fever or rheumatoid arthritis (A.S.T. 50).

An analysis of the 39 cases showed that 14 patients had previously experienced a minimum of one rheumatic attack. The antistreptolysin titres in these cases ranged from 166 units to 1250 units with an average titre of 625 units.

TABLE XII

Group I. Rheumatic Fever.

Age Group	No. of Cases	Mean A.S.T.	A.S.T. Distribution												
			<12	12	50	100	125	166	250	333	500	625	833	1250	2500
0 - 5	3	736					1						1	1	
6 - 10	8	594						1	2	1			3	1	
11 - 20	13	513			1			2	1		1	2	4	2	
21 & over	15	587			2			2	3	1		1	5		1
Total	39	519			3		1	5	6	2	1	3	13	4	1

Group II. Glomerulonephritis.

This group (Table XIII) of ten cases was too small for analysis in age groups. A mean antistreptolysin O titre of 396 units was found with a spread from 50 to 833 units. Two cases had antistreptolysin O titres below 166 and 8 cases had titres of 166 or over. Of the cases with 166 units or more, 4 were diagnosed as acute glomerulonephritis, 2 as acute nephritis and 2 as nephritis. The 2 cases with titres below 166 units were diagnosed as chronic glomerulonephritis.

TABLE XIII

Group II. Glomerulonephritis.

No. of Cases	Mean A.S.T.	A.S.T. Distribution												
		<12	12	50	100	125	166	250	333	500	625	833	1250	2500
10	396			1		1	2		2	1	1	2		

Group III. Other Streptococcal Diseases.

This group includes those diseases in which the streptococcus is usually the infecting agent. Fourteen cases were investigated. The mean titre was 475 units per ml. varying from 50 - 1250. Results are shown in Table XIV. The 4 cases of otitis media in this group had antistreptolysin titres of 250, 500, 625 and 1250 units respectively. Patients with pharyngitis and tonsillitis varied widely in antistreptolysin titres. In this group no attempt was made to investigate the specific bacterial aetiology of each illness and therefore cases of non-streptococcal aetiology may well be included.

TABLE XIV

Group III. Other Streptococcal Diseases.

Age Group	No. of Cases	Mean A.S.T.	A.S.T. Distribution												
			<12	12	50	100	125	166	250	333	500	625	833	1250	2500
0 - 5	3	510			1				1					1	
6 - 10	3	430						1			1	1			
11 - 20	4	733				1				1				2	
21 & over	4	219					1	1	1	1					
Total	14	475			1	1	1	2	2	2	1	1		3	

Group IV. Non-streptococcal Diseases

Fifty-three cases were investigated in which the diagnosis was some disease other than those in which the streptococcus is usually incriminated. The results are shown in Table XV. The mean antistreptolysin titre for the group was 94 with a range from 12 to 500 units. In the 0 - 5 age group the mean antistreptolysin titre was 46 units with a rise to 99 in the 6 - 10 age group and to 127 in the 11 - 20 age group. In the 21 and over age group the mean was 95 units, and in those in which the age was not revealed the mean titre was 69 units.

Six cases were found to have antistreptolysin titres of 250 or over. The diagnoses of these cases were as follows: -

1. Influenza or psychoneurosis. (Aching joints and swollen knees.) A.S.T. 500.
2. Acute gastritis. A.S.T. 333. (Scarlet fever 2 years ago.)
3. Recurrent polyarthritis. A.S.T. 333.
4. Abdominal pains. A.S.T. 333.
5. Alveolar abscess with arthritic pain. A.S.T. 250.

6. Congenital heart disease. Superimposed rheumatic fever? A.S.T. 250.

These high titres together with the history and diagnoses of most of these cases can be attributed to a possible streptococcal aetiology.

TABLE XV

Group IV. Non-streptococcal Diseases.

Age Group	No. of Cases	Mean A.S.T.	A.S.T. Distribution												
			< 12	12	50	100	125	166	250	333	500	625	833	1250	2500
0 - 5	7	46	2	1	1		1	1	1						
6 - 10	4	99	1	1	1					1					
11 - 20	11	127	3		1	1	4		1		1				
21 & over	26	95	3	1	10	3	4	3		2					
unknown	5	69	2	1				2							
Total	53	94	11	4	13	4	9	6	2	3	1				

Group V. Mothers and Newborn Infants

In the investigation of 31 maternal sera and 31 cord sera of newborn infants the average antistreptolysin O titres found were 93 units for mothers and 143 units for infants. The distribution of titres is shown in Table XVI.

In the comparison of each maternal serum to the corresponding infant serum it was found that 5 sets had identical titres, 24 sets had higher titres in the cord, and 2 sets had lower titres in the cord sera.

There were 3 mothers with titres higher than 166 units, the limit for normal sera. No evidence was available on possible strepto-

coccal infections in these people.

TABLE XVI

Group V. Mothers and Newborn Infants.

Group	No. of Cases	Mean A.S.T.	A.S.T. Distribution												
			<12	12	50	100	125	166	250	333	500	625	833	1250	2500
Maternal	31	93	3	5	8	5	4	3	1	2					
Cord	31	143		2	8	3	9	4	3	1			1		

DISCUSSION

The appearance of antistreptolysin O in the blood serum of humans is an almost inevitable immunological response to hemolytic streptococcus infection. This is of little clinical significance in patients where the streptococcal infection can be determined by direct bacteriological investigation, but in patients suffering from certain sequelae of streptococcal disease where infection may not be detectable or even no longer present antistreptolysin titration may be the important clue to the diagnosis. Many workers have stressed the significance of such titrations in the differential diagnosis of rheumatic and rheumatoid disorders and in determining the aetiology of glomerulonephritis. Yet certain difficulties have interfered with the adoption of the test as a routine laboratory procedure.

Chief among the difficulties has been the maintenance of stable preparations of streptolysin O. Without such preparations few laboratories have been in a position to prepare and standardize fresh streptolysin every time a test was requested. Methods of maintaining the stability of streptolysin O have been investigated and it has been shown that the substance can be kept as a fluid under vaseline seal in the refrigerator for many months, or alternatively in the desiccated form. During this investigation dehydrated streptolysin O has been produced commercially and is now on the market. Thus one may anticipate a greater clinical demand for antistreptolysin titrations and a great similarity of the test in the average laboratory.

Most people have been infected with hemolytic streptococci at some time in their lives, often on repeated occasions, and the aetiology of many of these infections, particularly those of a minor respiratory nature, may never have been determined or even suspected. Consequently most people can be expected to have antistreptolysin in their blood serum in amounts varying according to the degree, frequency or proximity of such infections. Fortunately it appears reasonably well established that titres developing in rheumatic fever and in glomerulonephritis are much higher than those encountered in an average population. This dependence on the measurement of titres for clinical significance emphasizes the necessity for using standard units. Such units were suggested by Todd and have been accepted by most subsequent workers; their suitability has probably now been confirmed by their adoption in relation to the commercial preparation referred to above. There still remains to be determined the uniformity or otherwise of such titrations from area to area and the possibility that different basic communal antibody levels may be encountered and have some bearing on the titres in diagnostically important infections.

With regard to rheumatic fever it was hoped that some cases might be followed from the beginning of the streptococcal infection to the occurrence of rheumatic fever and on into convalescence with the determination of antistreptolysin O titres at frequent intervals. Unfortunately clinical cooperation was insufficient to support this ideal, and it proved impossible to obtain specimens even at comparable periods of disease. Thus it is unexpectedly gratifying to find how closely some of these results compare with those of other workers.

The mean titre of 519 units found in the rheumatic fever group is very similar to the 500 units found by McCarty (1952). Rantz, Di Caprio and Randall (1952) found mean titres of 398 for children and 553 for adults suffering from rheumatic fever. The significance of individual titres, however, was not always clear. There was a close association between the finding of high titres and clinical rheumatic fever, but no absolute correlation of any antistreptolysin findings with clinically equivocal cases. Most of the latter had quite low titres and these might have indicated the community antibody level or the fall described by McCarty as occurring in convalescence from the acute phase of rheumatic fever. The findings, however, left the impression that high titres, even single examinations, were likely to be significant whereas low titres in single cases had little meaning.

In cases of acute glomerulonephritis Rantz, Di Caprio and Randall (1952) found a mean titre of 887 units. In this investigation the mean titre was comparatively low (576 units). A higher value for the mean titre would be obtained if only acute glomerulonephritis cases were considered as had been done by Rantz and co-workers. From the few cases in this group it appears that the antistreptolysin titre is not elevated in chronic glomerulonephritis cases. The very low titres, under 12 units, found in the two cases of nephrosis conforms with similar results of Rantz, Di Caprio and Randall (1952). The phenomenon is interesting and suggests a common immunological peculiarity in such patients.

The possibility of high titres being due to an anamnestic reaction will always occur to one when dealing with such a commonly occurring antibody as antistreptolysin. Apparently this is unlikely. In the group of cases representing the non-streptococcal diseases, the

mean antistreptolysin O titre of 94 was identical to that found in presumably normal mothers. This certainly suggests that the antistreptolysin O titre does not rise in diseases other than streptococcal.

The only children examined were hospital patients, but as suggested above, those in the non-streptococcal diseases group can be considered equivalent to normals. In this group the patients in age group 0 - 5 years showed the lowest average titre (46 units). Similar findings were disclosed by Wilson, Wheeler and Leask (1934) who found an average titre of 50 units for the 1/4 - 3 age group. This titre was considerably below that found in newborn infants, an average of 143 units. The last figure is somewhat higher than that of Gordon and Janney (1941) but bears a similar ratio to the mothers' titres as was found by these workers. Their conclusions that the titre dropped in a few months after birth to a very low level and gradually rose until the age of 10 - 19 when it approximated that at birth were confirmed in this investigation.

The high antistreptolysin titre in babies is interesting but seems to be related to a common phenomenon. Barr, Glenny and Randall (1949) noted that diphtheria antitoxin titres were higher in newborn infants than in their mothers. The difference in antistreptolysin levels between 93 in the 31 mothers examined and 143 in their infants is obviously due to some similar physiological phenomenon. Whatever the explanation, the transference of an increased ratio of antibody globulin from the maternal to the infantile bloodstream is undoubtedly of great protective value during the dangerous neonatal period.

SUMMARY

A technique for titrating antistreptolysin in serum has been described. It follows very closely the method employed by Rantz and Randall (1945) but requires a smaller volume of serum, a convenience when testing specimens from very young children.

The investigation of 116 cases shows that high antistreptolysin O titres can be expected in most rheumatic fever cases and in glomerulonephritis cases. Mean values found were 519 units for the rheumatic group and 396 units for the glomerulonephritis group. These values would be much higher if only the acute cases were considered. Patients suffering from diseases in which streptococci are usually incriminated showed a mean titre of 475 units. Those patients suffering from diseases other than streptococcal gave a mean titre of 94 units which was very close to the titre found in the sera of normal mothers. Newborn infants were found to give titres somewhat higher than those found in mothers.

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